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(54) Title: METHOD FOR MAKING GAMMA GLOBULIN-CONTAINING COMPOSITIONS

(57) Abstract

Gamma globulin concentrates are treated with ion exchange resins ultrafiltered in the presence of a stabilizer to inhibit the generation of anticomplement activity during such treatment or ultrafiltration.

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METHOD FOR MAKING GAMMA GLOBULIN-CONTAINING COMPOSITIONS

This invention relates to the ultrafiltration and ion exchange treatment of gamma globulin-containing compositions. In particular, this invention is concerned with inhibiting the generation of anticomplement activity during the membrane ultrafiltration and/or ion exchange treatment of gamma globulin concentrates.

Gamma globulin concentrates are compositions comprising immune globulin G and other proteins wherein the proportion of gamma globulin is greater than that found in normal pooled human plasma. The concentrates are well known and may be obtained by fractional precipitation of pooled plasma by alcohols and the like.

It is frequently desirable to ultrafilter such concentrates to remove salts, biologically active polypeptides and other undesired small molecules, as well as to concentrate protein in the solutions. Conducting ultrafiltration to remove small molecules without concommitantly increasing the concentration of protein is also known as dialysis. For the purposes herein, membrane ultrafiltration will be considered to include any procedure in which relatively small molecules in a solution or suspension are separated from comparatively large molecules by forcing or allowing the small molecules to pass through a membrane or barrier (such as a gel) having a pore size which is too small to allow the large molecules to pass through the membrane or barrier. Gamma globulin concentrates are generally

ultrafiltered by the use of commercially available cassettes having membranes which pass molecules of less than about 100,000 Daltons. The concentrates can be dialyzed by exchanging dialysis fluids against bags constructed of the same membranes and holding the concentrates to be dialyzed.

Habeeb et al. ("Vox Sang." 32:143-158 [1977]) teach dialyzing a solution of Cohn Fraction II (a plasma fraction containing elevated gamma globulin levels obtained by ethanol precipitation), passing the solution through a DEAE (diethylaminoethyl)-cellulose column to adsorb undesired proteins from the dialyzed solution, followed by recovering the enriched gamma globulin eluate. According to the authors, chromatography on DEAE-cellulose resulted in low levels of gamma globulin aggregates and anti-complement activity in the final immunoglobulin product.

It has been an objective of the art for quite some time to remove anticomplement activity. This activity has made it diffucult to safely inject gamma globulin products intravenously since such injections frequently result in severe side effects. The alternative has been to inject the products intramuscularly, but this reduces the therapeutic efficacy of the gamma globulin.

In applicant's view, ultrafiltration and, to a lesser extent, chromatography of gamma globulin concentrates on anion exchange resins results in products having unacceptably high anticomplement activity. Accordingly, it is an object of this invention to employ chromatography on ion exchange resins and/or ultrafiltration in manufacturing gamma

globulin products, whil also r ducing or preventing th g neration of anticomplem ntary activity in such products. This and other objects f th invention will become more apparent from consideration of this specification as a whole.

SUMMARY OF THE INVENTION

The anticomplement activity of gamma globulin products made by methods comprising ultrafiltration and/or treatment with ion exchange resins is considerably reduced by including one or more substantially non-surface-active stabilizers with gamma globulin concentrates during such ultrafiltration and/or ion exchange resin treatments. Heretofore it has not been believed helpful or necessary to include stabilizers with gamma globulin during such steps, so it was particularly surprising to find that such stabilizers are necessary for the production of gamma globulin having acceptably low levels of anticomplement activity.

DETAILED DESCRIPTION OF THE INVENTION

The starting gamma globulin concentrates are gamma globulin containing fractions obtained from plasma, tissue, recombinant microbial cultures or other sources. Suitable starting concentrates, if obtained from human plasma, will have a gamma globulin concentration per unit weight of protein that is higher than that found in normal pooled human plasma. Such concentrates generally contain greater

than about 80%, preferably greater than about 96% gamma globulin by weight of total protein, the remaining protein including albumin, prothrombin complex and other plasma constituents. Cohn fractions such as fraction II are well known and suitable starting concentrates for ultrafiltration and ion exchange treatments. The concentrates are preferably essentially free of protease hydrolysis fragments of gamma globulin.

The concentrate may contain a high titer for a particular antigen or class of antigens of interest. This means that the concentrate will have a greater proportion of antibodies specific for such an antigen or class of antigens than is found in pooled normal plasma. Such "hyperimmune" globulin concentrates will usually contain high titers for various cellular or viral pathogens such as Clostridium or hepatitis.

The stabilizers to be used herein are substances which inhibit the generation of anticomplement activity which occurs during ultrafiltration or ion exchange treatment of gamma globulin concentrates. Whether or not a given substance is effective for this purpose is easily determined by conducting the ultrafiltration and/or ion exchange treatment of the immunoglobulin-containing starting material in the presence and absence of the substance, and thereafter determining the anticomplement activity of the treated materials. If the treated material with the test substance has an anticomplement activity which is at least about 20% less than that of the treated material without the test substance, then such substance exerts the desired activity.

The stabilizers preferably are physiologically acceptable or are used in such amounts that the final product contains a physiologically acceptable quantity of the stabilizer. This means that the stabilizer should be substantially non-surface-active because soaps, detergents, surfactants and other highly polar materials can be harmful to the blood cells of patients. Otherwise, an additional step will be required to remove or reduce the concentration of the stabilizer before the product concentrate can be administered to patients; this step can be expensive and time-consuming.

Suitable stabilizers generally fall into three groups: Hydrophilic macromolecules, amino acids and low molecular weight polyols.

The hydrophilic macromolecule usually will be a polymer, and is desirably a polymer which can be metabolized completely or digested to innocuous fragments in the patient's circulation and/or excreted. Such polymers will have an average molecular weight greater than about 1000 Daltons, preferably about from 3000 to 50,000 Daltons. As a practical matter, polymers are infrequently available in which every molecule is of the same molecular weight. Accordingly, molecular weights disclosed herein shall be considered average, with the actual molecular sizes ranging plus or minus up to about 30%.

The macromolecule preferably is non-proteinaceous. The reason for this is that synthetic proteinaceous amides or proteins from nonhuman sources are frequently antigenic upon administration to patients, and proteins from human sources are

comparatively ineffective in preventing the generation of anticomplement activity during ultrafiltration and/or in exchange treatments.

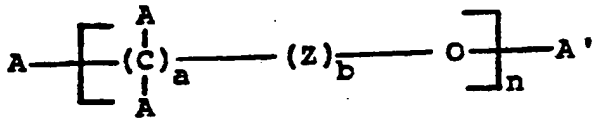
The macromolecule should be sufficiently water soluble to stabilize the concentrates. This means that the macromolecule is sufficiently hydrophilic to go into solution at a concentration of at least about 3% weight/volume in saline at room temperature. Obviously, this solubility requirement may vary depending upon the concentration of protein in the concentrate solution; lower protein concentrations will require less stabilizer, and thus the solubility can be lower. Saturated solutions may be used, and slight turbidity imparted by colloidal particles of excess water insoluble macromolecule is tolerable if the particles are not of a size to be a hazard to patients. However, it is preferred that the macromolecule be readily and completely water soluble at least in the amounts required for adequate stabilization.

This invention contemplates adding the macromolecule to gamma globulin concentrates as a solid or as a predissolved solution. It may be necessary to heat or otherwise treat the macromolecule to form a solution before it is combined with the concentrate. In such a case the macromolecule should not gel, precipitate or crystallize upon cooling or cessation of the dissolution treatment.

Suitable macromolecules ordinarily will fall within three general classes: polyethers, polysaccharides and hydrophilic vinyl polymers.

Generally the first class is preferred for maximal gamma globulin stability, with polysaccharids being most preferred for physiological acceptability.

Suitable polyethers are generally polythers synthesized from hydroxylated monomers. Such polyethers are embraced within polymers having the following general structure:



wherein A' is H, A is H, -OH, $-NH_2$, $-CH_2OH$, $-CH_2NH_2$, or $-CH_2COOH$, a is 1 to 3, n is greater than about 20, Z is $-CH_2-$, b is zero or 1; and block copolymers of such polymers. Polyethylene glycol is the preferred polymer.

The polysaccharides which may be employed include branched and unbranched polymers (n>3) of five and/or six carbon sugars, including such sugars as ribose, xylose, mannose, glucose, galactose and fructose, and derivatives thereof. Exemplary polysaccharides are starch, glycogen, hydroxyethyl starch, polyglucose, dextran, xylan, pectin, acacia and hydrolysates thereof.

The hydrophilic vinyl polymers are polyhydroxy-substituted or carry other hydrophilic substituents. Examples include polyvinyl alcohol or polyvinylpyrrolidone.

The amount of macromolecule to be used is subject to some discretion. The optimal quantity should be determined by routine experimentaion, but it will

generally range in a weight ratio to the total protein present in the gamma globulin concentrate of about from 0.0075 to 0.062, preferably about from 0.01 to 0.05. This quantity should be more than the trace or residual levels remaining after a precipitation step in which gamma globulin is precipitated from a solution containing the macromolecule. Thus, even if the macromolecule is present in solutions from which gamma globulin is precipitated as part of an earlier purification procedure, a supplementary amount of the macromolecule should be added to the redissolved, precipitated protein before it is ultrafiltered or treated with ion exchange resins.

Particularly beneficial results are obtained by using the macromolecule in concert with the two other groups of gamma globulin stabilizers: amino acids and low molecular weight polyols. The use of one or more of such stabilizers along with the hydrophilic macromolecule permits the use of less of each stabilizer than would have been the case if any one stabilizer had been employed alone.

The polyol is a compound having a molecular weight of less than about 1000 Daltons and a high degree of substitution by hydroxyl groups, generally up to about seven hydroxyl groups per molecule. The preferred polyols are sugar alcohols or reducing and nonreducing mono, di and trisaccharides. Exemplary polyols include mannitol, sorbitol, glucose, mannose, lactose, fructose and maltose. Glucose is most preferred.

The amino acid is generally an aliphatic or neutral amino acid, i.e., leucine, isoleucine, valine or glycine. Glycine is preferred.

The amount of polyol or amino acid to be used shall be determined in the same fashion as described above for the macromolecule, although typical amounts for polyol and amino acid range respectively in a weight ratio to total protein in the concentrate from about 0.05 to 1.25, preferably 0.1 to 0.5 and from about 0.050 to 0.6, preferably 0.075.

Ultrafiltration of gamma globulin concentrates may precede or follow ion exchange treatment. When ultrafiltration precedes the ion exchange step, it is generally conducted as dialysis for purposes of removing undesired molecules, generally of small molecular weight such as salts or solvents. Ultrafiltration after ion exchange treatment is frequently to increase the protein concentration prior to lyophilization or for storage. Gamma globulin stabilizers are useful in either situation, or in ultrafiltration and ion exchange adsorption alone.

The concentration of total protein in concentrates for dialysis ultrafiltration is generally about from 30 to 60 g/L, of which about 90% by weight is gamma globulin. The solution to be ultrafiltered is placed in a dialysis membrane bag, such membranes having the capacity to pass molecules of less than about 100,000 Daltons. The bag is then immersed in a circulating dialysis fluid. Small molecules will diffuse into the dialysis fluid, which is customarily drawn off and substituted with fresh fluid as the dialysis progresses. The dialysis is stopped when the desired concentration of small molecule is reached in the dialysis retentate. This is a function of the identity of the small molecules

as well as what fractionation steps, if any, are to follow the dialysis. Thus the dialysis time, temperature and other parameters will be optimized in each case as required.

Ultrafiltration concentration is used to increase protein (and thus gamma globulin) concentration. It can also be used in a dialysis mode by adding dialysis fluid to the concentrate solution and continuing to ultrafilter until a higher, desired protein concentration is reached, usually about from 4 to 8 percent protein weight/volume, during which the undesired small molecules also are removed.

Ultrafiltration dialysis or concentration is accomplished on a large scale by the use of commercially available ultrafiltration membrane systems. These are devices in which the geometry of the ultrafiltration system has been optimized for the most efficient removal of water and/or small solute molecules from protein solutions, including directing the retentate flow laterally across the membrane surface to reduce clogging of the filter membrane pores by retained proteins.

In accordance with this invention the solution of gamma globulin concentrate will contain one or more stabilizers in the amounts described above. If the ultrafiltration membrane has a molecular weight cut-off higher than that of the stabilizer then preferably any dialysis solution will contain a similar concentration of stabilizer as to prevent a reduction in stabilizer concentration during treatment.

Methods for purifying gamma globulin concentrates by the use of ion exchange resins are known. For

xample, see R if, "Immunochemistry" 6:723-731 (1969), Hoppe et al., "Vox Sang." 25:308-316 (1975) and U.S. Patent 4,312,949. Ion xchang r sins are water insoluble materials substituted with positiv ly or negatively charged ionic groups, or mixtures of such groups (amphoteric resins). They are generally granular and have been equilibrated before use with a physiologically acceptable ion such as chloride, hydroxyl or alkali metal ions. The resins are selected to adsorb desired or undesired proteins from admixture with the target protein. Selection and use of appropriate resins is within the skill of the ordinary artisan. Generally, an anion exchange resin such as DEAE-cellulose is used in the purification of globulin concentrates because it is capable of preferentially adsorbing prothrombin complex proteins from the concentrate.

The solution of gamma globulin concentrate is recovered by centrifuging or filtering to remove the insoluble resin and its bound protein. The ion exchange adsorption process can be repeated several times. In accordance with this invention, the solution of gamma globulin concentrate which is incubated with the ion exchange resin will contain one or more stabilizers in the amounts described above.

It is possible to perform an ultrafiltration step simultaneously with the ion exchange procedure. This is readily accomplished by simply mixing an ion exchange resin with the concentrate solution and ultrafiltering the resulting mixture. Generally, however, it is preferred that the ultrafiltration and ion exchange treatments be sequential, with ultra-filtration dialysis preceding ion exchange treatment.

It is not necessary to separate the stabilizers from the gamma globulin concentrate following ultrafiltration and ion exchange treatments sc long as the stabilizers are physiologically acceptable in the amounts employed. Thus, in the broad preferred embodiment, a solution of Cohn Fraction II or other concentrate is dissolved in a solution having a pH of about from 5.0 to 5.6, usually 5.3, which contains polyethylene glycol at a concentration ranging from about 0.05% (w/v) to just below that at which proteins in the concentrate will precipitate, generally about 5-8% (w/v). This is dialyzed against about 3 to 5 volumes of a solution ocntaining about half as much polyethylene glycol (w/v) as is present in the concentrate solution. The dialysis retentate is recovered and the pH of the concentrate adjusted to about 8.0 to optimize the ion exchange adsorption to follow. Hydrated DEAE Sephadex is mixed with the concentrate solution to adsorb undesired protein and the DEAE Sephadex then separated from the concentrate. A polyol, amino acid and albumin are added in amounts sufficient to inhibit the generation of anticomplement activity during lyophilization, the concentrate sterile filtered, filled into containers, lyophilized and the containers hermetically sealed. This product contains less than 200 Ch50 units of

anticomplementary activity/gram of protein. Without the use of polyethylene glycol during the dialysis and ion exchange adsorption steps the anti-complementary activity/gram of protein is frequently over 10 times higher.

EXAMPLE

Dialysis was performed in a Millipore dialysis system which consisted of two Millipore Pellicon® cassette holders (stainless steel), twenty Millipore PTHK cassettes (100,000 Dalton cut-off), and two diaphragm pumps. Prior to dialysis, the system was washed to remove bacterial pyrogens. After depyrogenation, the system was washed with several liters of cold dialysis buffer. The dialysis procedure was performed at 5°C. Dialysis flow rates and pump speeds were adjusted to avoid the generation of foam. All solutions were prepared using pyrogen-free water.

An aqueous starting solution of Cohn Fraction II at a protein concentration of 50g/liter and having a pH of 5.3 was dialyzed against 4 volumes (approximately 300 liters) of a solution which contained 20 mM NaCl and 0.5 g/L PEG-4000. The Fraction II solution was pumped through the Millipore cassettes where materials with a molecular weight of less than 100,000 Daltons were removed by ultrafiltration. The retentate, which contained concentrated immune globulins (150,000 Daltons and up), was returned to and mixed with the immune globulin solution undergoing dialysis. Simultaneously, the above described dialysis solution

was continuously pumped into th immune globulin solution at the same rate as filtrat was produced and removed via the cassettes.

The immune globulin solution was stirred constantly to distribute the retentate and the continuously added dialysis solution. By adjusting the pump supplying the dialysis solution and the pump feeding the mixed retentate and dialysis solution into the cassettes, the volume and protein concentration of the immune globulin solution remained constant. When the 300 liters of dialysis solution was depleted, the immune globulin solution was concentrated to a protein concentration of 55 mg/ml using the cassettes in conventional fashion.

The concentrated immune globulin solution was treated with an ion exchange resin in the following fashion. Tris base was added to the solution to produce a final tris concentration of 0.025M and the pH was adjusted to 8.0 $\pm$ 0.1. Hydrated DEAE (diethylaminoethyl) Sephadex A-50 was mixed with the solution at a concentration of 5 g/g of protein and the mixture was agitated for 3 hours at 5°C, whereafter the Sephadex A-50 was filtered from the solution. NaCl, dextrose, glycine and albumin were added to the solution to make final concentrations, respectively of 0.85%, 2.0%, 2.25% and 0.1%, pH was adjusted to 7.0 and the protein concentration adjusted to 5.2%. This solution contained about 1.3 mg of PEG-4000/ml, carried over from the dialysis step. The solution was sterile filtered, filled into vials, lyophilized and the vials sealed.

WE CLAIM:

1. In a method for preparing gamma globulin comprising ultrafiltering and/or treating with an ion exchange resin a gamma globulin concentrate, the improvement comprising including at least one stabilizer with a gamma globulin concentrate during ultrafiltration and/or during treatment with the ion exchange resin, said stabilizer being included in an amount sufficient to reduce the anticomplement activity of the gamma globulin so prepared.

2. The method of Claim 1 wherein the gamma globulin concentrate contains greater than about 80% gamma globulin by weight of total protein in the concentrate.

3. The method of Claim 1 wherein the stabilizer is a hydrophilic macromolecule, a low molecular weight polyol or an amino acid.

4. The method of Claim 3 wherein the macromolecule is a polyether, polysaccharide or hydrophilic vinyl polymer.

5. The method of Claim 4 wherein the polyether is polyethylene glycol.

6. The method of Claim 4 wherein the stabilizer is not a protein and is substantially non-surface-active.

7. The method of Claim 1 wherein the ultrafilt ring is conducted as to increas the weight of concentrate per volume of concentrate solution.

8. The method of Claim 1 wherein ultrafiltering precedes an ion exchange treatment.

9. The method of Claim 1 wherein the ultrafiltering comprises dialyzing the solution of gamma globulin concentrate against at least four volumes of dialyzing solution.

10. The method of Claim 9 wherein the dialyzing solution also contains said stabilizer.

11. The method of Claim 10 wherein the dialyzing solution is buffered.

12. The method of Claim 5 wherein the polyethylene glycol has an average molecular weight of about 4000 Daltons.

13. The method of Claim 1 wherein the ultrafiltering follows the ion exchange treatment.

14. The method of Claim 8 wherein the stabilizer is included during both ultrafiltering and ion exchange treatment.

15. The method of Claim 8 wherein the ion exchange treatment comprises adsorbing protein from

the concentrate onto an anion exchange r sin and recovering the gamma globulin containing, unadsorbed prot in.

16. The method of Claim 4 wherein the macromolecule is a polyglucose.

17. The method of Claim 3 wherein the stabilizer is a macromolecule having a molecular weight greater than about 1000 Daltons.

18. The method of Claim 15 which further comprises recovering a solution of the gamma globulin containing protein, filtering the solution to retain cellular micro-organisms, filling the solution into containers, lyophilizing the solution and hermetically sealing the containers.

19. The method of Claim 1 wherein the concentrate is an alcohol precipitate of gamma globulin-containing protein from human plasma.

20. The method of Claim 1 wherein the ultra-filtration is conducted at a pH of about from 5.0 to 5.6.

21. The method of Claim 3 wherein the macro-molecule is substantially free of charged groups.

22. The method of Claim 4 wherein the macromolecule is present in a concentration insufficient to precipitate proteins present in the concentrate.

23. The method of Claim 5 wherein the polyethylene glycol is present in a concentration of about from 0.1% to 2.0% by weight/volume of concentrate solution.

24. The method of Claim 18 wherein the product of Claim 18 is reconstituted and administered intravenously.

25. The method of Claim 1 wherein a mixture of stabilizers is used.

26. The method of Claim 25 wherein the mixture comprises polyethylene glycol, a polyol and an aliphatic amino acid.

27. The method of Claim 1 wherein the concentrate is a solution of Cohn Fraction II.

28. The method of Claim 1 wherein the stabilizer is free of hydroxyethyl starch.

INTERNATIONAL SEARCH REPORT

International Application No PCT/US83/01016

I. CLASSIFICATION F SUBJECT MATTER (if several classification symbols apply, indicate all) [3]

According to International Patent Classification (IPC) or to both National Classification and IPC

INT. CL.[3] A61K 39/395; A61K 39/00; A61K 35/14

II. FIELDS SEARCHED

Minimum Documentation Searched [4]

Classification System	Classification Symbols
U.S.	260/112B; 424/85, 424/101; 424/176 424/177

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [5]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [14]

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No. [18]
P,Y	US, A, 4,384,993, PUBLISHED 24 MAY 1983 SATO ET AL	1-28
E,Y	US, A, 4,396,608, PUBLISHED 02 AUGUST 1983 TENALD	1-28
P,Y	US, A, 4,374,763, PUBLISHED 22 FEBRUARY 1983 TAKAGI	1-28
Y	US, A, 4,276,283, PUBLISHED 30 JUNE 1981 EIBL ET AL	1-28
Y	US, A, 4,136,094, PUBLISHED 23 JANUARY 1979 CONDIE	1-28
Y	US, A, 4,093,606, PUBLISHED 06 JUNE 1978 COVAL	1-28

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"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search [2]	Date of Mailing of this International Search Report [2]
19 AUGUST 1983	31 AUG 1983
International Searching Authority [1]	Signature of Authorized Officer [20]
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